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(54) Title: CULTURE MEDIUM WITH YEAST OR SOY BEAN EXTRACT AS AMINOACID SOURCE AND NO PROTEIN COMPLEXES OF ANIMAL ORIGIN (57) Abstract The invention relates to a medium for culturing pathogenic bacteria to produce an immunogenic factor. The medium comprises non-animal derived proteinaceous material. The invention also relates to the use of the medium to cultivate pathogenic bacteria, obtaining immunogenic factors from the bacteria being cultivated and preparing vaccines using the immunogenic factors.		

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CULTURE MEDIUM WITH YEAST OR SOY BEAN EXTRACT AS AMINOACID SOURCE AND NO PROTEIN COMPLEXES OF ANIMAL ORIGIN

The present invention relates to a medium for cultivating pathogenic bacteria. The present invention also relates to the use of the medium to cultivate pathogenic bacteria, obtaining immunogenic factors from the bacteria being cultivated and preparing vaccines using the immunogenic factors.

Bacterial vaccines are produced by cultivating pathogenic bacteria in a medium, isolating immunogenic factors and preparing vaccines based on the isolated immunogenic factors.

10 Such methods are described in Bacterial Vaccines, 1984, Ed. Rene Germanier Academic Press and Bacterial Vaccines in Advances in Biotechnology Processes, Vol. 13, 1990, Ed. A. Mizrahi, Wiley-Liss. In conventional methods, the pathogenic bacteria are cultivated in media containing proteinaceous material of animal origin. These compounds are used in the belief that some growth factors, essential for the growth of

15 pathogenic bacteria, were only present in compounds of animal origin such as blood, brain heart infusion, meat, etc. For instance, *C. tetani* is grown in media containing a heart infusion and an enzymatic digest of casein; *C. diphtheriae* requires a beef infusion; *H. pylori* is grown in media containing peptamin and tryptone; and *Haemophilus influenzae* is grown in media containing proteose peptones. World Health Organisation

20 report series numbers 800 (1990) and 814 (1991) indicate that to grow *Haemophilus influenza*, *Corynebacterium diphtheriae*, *clostridium tetani* and *Bordetella pertussis*, media comprising compounds of animal origin are required.

The requirement for proteinaceous material of animal origin in the media gives rise to

25 concern over possible contamination of the media. In particular, concern that the media may be contaminated with the bovine spongiform encephalopathy (BSE) causative agent or other infectious and harmful agents, restricts the usefulness of any factors derived from such cultures, especially in therapeutic applications.

30 It has surprisingly been found that proteinaceous materials of non-animal origin are able to sustain the growth of pathogenic bacteria and enable the production of immunogenic factors by the bacteria.

In patent application DD 294 502-A, a process for preparing soya hydrolysates and the use of the soya hydrolysates in culturing microorganisms for fimbriae is disclosed. The soya hydrolysate is prepared by culturing soya-flour-containing medium with
5 streptomyces strains. The soya hydrolysate is used as a carbon source due to the high percentage of polysaccharides present and contains less than 20% by dry weight protein.

The use of proteinaceous material of non-animal origin, such as proteins from soy beans, cotton seeds, potatoes, etc., as a media constituent for the cultivation of pathogenic
10 bacteria, completely removes the risk of animal derived contamination, such as BSE, being transmitted into humans in any subsequent therapeutic or prophylactic applications.

A further advantage associated with the use of vegetable derived proteinaceous materials
15 is the reduction in cost of producing the materials and the increased consistency in the materials (non-animal derived proteinaceous material is more uniform in its composition than animal derived materials).

The present invention provides a medium for cultivating pathogenic bacteria to produce
20 an immunogenic factor wherein the medium comprises at least 20% by dry weight of a non-animal derived proteinaceous material, and does not comprise animal derived proteinaceous material.

Any standard medium for cultivating bacteria may be used as the basis for the medium
25 of the present invention, provided the medium does not contain animal derived proteinaceous material.

Preferably, the medium of the present invention comprises a carbon and an energy source, a nitrogen source, essential salts and optionally a selecting agent, such as an
30 antibiotic, for selecting the microorganisms to be cultured.

The medium of the present invention may be a solid or liquid medium. Examples of

standard liquid media which may form the basis of the medium of the present invention include Brucella Broth (without tryptone and peptamine), Watson medium (without casamino acids), Mueller Miller medium (without heart infusion and casein hydrolysate), CL medium (without casamino acids) and Franz A medium. Standard solid media can
5 be prepared from any of the liquid media by the addition of a solidifying agent such as agar.

The term "cultivating" as used herein means the maintenance of, and preferably the growth of, bacteria. Bacterial growth is herein defined as an increase in bacterial
10 biomass.

The term "pathogenic bacteria" as used herein, means any bacteria which is involved in the pathogenesis of a disease. Preferred pathogenic bacteria include *Helicobacter pylori*, *Haemophilus influenzae*, *Corynebacterium diphtheriae* and *Neisseria meningitidis*,
15 *Bordetella pertussis* and *Clostridium tetani*.

The term "immunogenic factor" as used herein, means any factor which is capable of stimulating the immune system of a human or animal. Such immunogenic factors include antigenic proteins and especially virulence factors and fragments thereof.
20 Virulence factors are defined as being associated with the virulence of a bacteria and includes such factors as the vaculating cytotoxin VacA produced by *Helicobacter pylori*. Other virulent factors are described by Rappuoli, R. et al., European Journal of Gastroenterology and Hepatology of *Helicobacterpylori* infection. Proceedings of an interdisciplinary meeting (Genova, June 18-19, 1993) J.J. Misiewicz, Ed. (CS Current
25 Science), pp S76-S78 (incorporated herein by reference). The immunogenic factor may be genetically detoxified or treated by a toxoiding process. Methods for genetically detoxifying and toxoiding immunogenic factors are well known to those skilled in the art and include those described by Rappuoli, R., Vaccine, 12, 579-581, (1994) (herein incorporated by reference).

The term "proteinaceous material", as used herein, means proteins and protein degradation products including free amino acids. Preferably, the proteinaceous material

is a protein hydrolysate.

Non-animal derived proteinaceous materials as used herein means proteinaceous materials derived from non-mammalian sources, such as vegetables, birds, fish, yeasts, 5 fungi, algae and microorganisms. More preferably, non-animal proteinaceous materials mean proteinaceous material derived from vegetables, yeasts, algae and microorganisms. Most preferably, non-animal derived proteinaceous materials means proteinaceous materials derived from vegetables such as protein compositions derived from soy beans, cotton seeds, potatoes, etc.

10

Preferred non-animal derived proteinaceous materials include yeast extracts such as HY YEST (Quest) and soy bean derived protein compositions such as Hysoy (Quest), Amisoy (Quest), N-Z soy (Quest) and Soytone (Difco).

15 Yeast extracts can be prepared by standard procedures well known to those skilled in the art. Furthermore, yeast extracts are commercially available from numerous sources including Sigma and Quest.

Soy bean derived protein compositions can be prepared by enzymatic digestion of soy 20 bean meal or soy isolate using standard enzymes such as papain. For example, N-Z soy is a soluble protein composition made by the enzymatic digestion of a soy isolate and Hysoy is a papaic digest of soy bean meal. Soy bean derived protein compositions can also be obtained by acid hydrolysis of a soy isolate. For example, Amisoy is a source of amino acids and peptides produced by acid hydrolysis of a soy isolate.

25

Other non-animal derived proteinaceous materials can be obtained by either enzymatic digestion or by acid hydrolysis of a protein containing material of a non-mammalian source.

30 The non-animal derived proteinaceous material in the medium of the present invention may comprise two or more different non-animal derived proteinaceous materials, such as a mixture of soy bean derived protein compositions such as Hysoy, Amisoy, N-Z soy

and Soytone.

Animal derived proteinaceous materials include protein compositions such as fetal calf serum (FCS), bovine serum albumin (BSA), proteose peptones, casamino acids, tryptone, peptamin and casein hydrolyzates.

Preferably, the medium of the present invention comprises at least 20% by dry weight of a non-animal derived proteinaceous material, more preferably, at least 30% by dry weight of a non-animal derived proteinaceous material and most preferably at least 50 % by dry weight of a non-animal derived proteinaceous material.

It has surprisingly been found that the cultivation of pathogenic bacteria using the medium of the present invention results in increased growth of the bacteria and an increased yield of immunogenic factors compared to cultivation of the pathogenic bacteria in a medium containing animal derived proteinaceous material.

The present invention further provides a process for making a medium for cultivating pathogenic bacteria to produce an immunogenic factor comprising adding sufficient non-animal derived proteinaceous material to a standard medium for cultivating bacteria, which does not comprise animal derived proteinaceous material, so that the medium for cultivating pathogenic bacteria comprises at least 20% by dry weight of the non-animal derived proteinaceous material, and does not comprise animal derived proteinaceous material.

Examples of standard liquid media include Brucella Broth (without tryptone and peptamine) Watson medium (without casamino acids), Mueller Miller medium (without heart infusion and casein hydrolysate), CL medium (without casamino acids) and Franz A medium. Standard solid media can be prepared from any of the liquid media by the addition of a solidifying agent such as agar.

30

The present invention further provides a culture comprising the medium of any of the previous claims and pathogenic bacteria. Preferred pathogenic bacteria include

Helicobacter pylori, *Haemophilus influenzae*, *Corynebacterium diphtheriae* and *Neisseria meningitidis*, *Bordetella pertussis* and *Clostridium tetani*. Most preferably, the pathogenic bacterium is *Helicobacter pylori*.

- 5 The present invention also provides a process for preparing an immunogenic factor of a pathogenic bacteria comprising the steps of cultivating the bacteria in the medium of the present invention and optionally purifying the immunogenic factor from the medium.

Preferably, the pathogenic bacteria are cultured in the medium of the present invention
10 for at least 6 hours, more preferably at least 36 hours, and most preferably at least 72 hours under suitable conditions for the production of the immunogenic factor.

Suitable culture conditions for the production of the immunogenic factor, including the duration of the culture, will vary depending on the bacteria being cultured. However,
15 one skilled in the art can easily determine the culture conditions required for the production of the immunogenic factor by following standard protocols, such as those described in the series *Methods in Microbiology*, Academic Press Inc., (incorporated herein by reference) and, if necessary, by performing a number of standard experiments to determine suitable culture conditions.

20

The immunogenic factor can be isolated from the bacterial culture using a number of standard techniques including those described by Manetti, R. et al., *Infect. Immun.*, 63, 4476-4480, (1995), incorporated herein by reference.

- 25 The present invention also provides a process for the production of a vaccine comprising preparing an immunogenic factor of a pathogenic bacteria comprising the steps of cultivating the bacteria in the medium of the present invention, optionally purifying the immunogenic factor from the medium and bringing said factor, optionally toxoided, into association with a pharmaceutically acceptable carrier. Suitable methods for producing a
30 vaccine are described by Rappuoli, R., *New and improved vaccines against Diphtheria and Tetanus*. (1990), 251-268, *New Generation of Vaccines*, Ed. G.C. Woodrow, M.M Levine, Marcel Dekker Inc. New York. (Incorporated herein by reference).

The vaccines prepared by the process of the present invention will require the addition of adjuvants when they are used. Suitable adjuvants are described in Gupta, R. K. et al., Vaccine, 13, 1263-1276, (1995).

5

The vaccines prepared by the process of the present invention can be used to vaccinate an individual against a bacterial infection. Preferred bacterial infections which can be vaccinated against include type B gastritis, bacterial meningitis, diphtheria, tetanus and whooping cough.

10

The vaccines prepared by the process of the present invention may be provided as a pharmaceutical composition comprising the vaccine of the present invention in admixture with a pharmaceutically acceptable carrier and adjuvants as mentioned above.

15 The vaccines prepared by the process of the present invention can be administered by oral or parenteral route, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), rectal and topical administration.

For oral administration, the compounds of the invention will generally be provided in the
20 form of tablets or capsules or as an aqueous solution or suspension.

Tablets for oral use may include the active ingredients (the vaccine component) mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavouring agents, colouring
25 agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and other well known agents, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl
30 monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

Capsules for oral use include hard capsules on which the active ingredient is mixed with

a solid diluent, and soft capsules wherein the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, the compounds of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The vaccines prepared by the process of the present invention may also be presented as liposome formulations.

The present invention is now described with reference to the following examples and to the figures in which:-

Figure 1 shows the kinetics of growth of *H. pylori* CCUG 17874 in BB containing Tryptone and peptamin.

20

Figure 2 shows the kinetics of growth of *H. pylori* CCUG 17874 in simplified BB containing Soytone.

Figure 3 shows the kinetics of growth *H. pylori* CCUG 17874 in simplified BB containing Hysoy.

Figure 4 shows a VacA immunoblot wherein lane 1 is molecular weight marker, lane 2 VacA standard 750ng, lane 3 VacA standard 500ng, lane 4 VacA standard 400ng, lane 5 VacA standard 150ng, lane 6 VacA standard 20ng, lane 7 VacA produced at the end of fermentation, lane 8 VacA produced after 48 hours of culture, lane 9 VacA produced after 30 hours of culture, lane 10 VacA produced after 23 hours of culture.

Figure 5 shows a VacA immunoblot wherein lane 1 is molecular weight marker, lane 2 VacA standard 750ng, lane 3 VacA standard 500ng, lane 4 VacA standard 400ng, lane 5 VacA standard 150ng, lane 6 VacA standard 20ng, lane 7 VacA produced at the end of fermentation, lane 8 VacA produced after 31.5 hours of culture, lane 9 VacA produced after 30 hours of culture, lane 10 VacA produced after 24 hours of culture.

Figure 6 shows a VacA immunoblot wherein lane 1 is molecular weight marker, lane 2 VacA standard 750ng, lane 3 VacA standard 500ng, lane 4 VacA standard 400ng, lane 5 VacA standard 20ng, lane 6 VacA produced at the end of fermentation, lane 7 VacA produced after 48 hours of culture, lane 8 VacA produced after 31 hours of culture, lane 9 VacA produced after 24.5 hours of culture, lane 10 VacA standard 150ng.

Figure 7 shows the kinetics of growth of *H. influenzae b* in simplified Franz medium containing Soytone or Proteose peptone.

15

Figure 8 shows the kinetics of growth of *N. meningitidis C* in Watson medium containing Casaminoacids or Amisoy.

Examples

20

All references are herein incorporated by reference.

Example I

25 *Helicobacter pylori* is a curved gram-negative microaerobic bacterium isolated about 10 years ago and is associated with type B gastritis in humans. This bacterium colonizes the human gastric mucosa and establishes a chronic infection that may result in gastric and duodenal ulcers (Blaser, M.J., (1990), J.Infect.Dis., 161, 629-633) and can be a risk factor for the development of gastric carcinoma (Parsonnet, J. *et al.*, (1991), New Engl. J. Med., 325, 1127-1131).

30

In the long term, the infection and the diseases could be prevented and treated by

vaccination. Currently, several factors involved in bacterial adhesion, colonisation and virulence have been identified. One of the most interesting factors involved in the disease is the vacuolating cytotoxin (VacA) that causes massive vacuolization in several mammalian cell lines (Leunk, R.D., (1991), Rev. Infect. Dis., 13(suppl.8), S683-S689).

- 5 Vacuoles have also been observed in the gastric epithelia of patients with chronic gastritis (Tricottet, V. *et al.*, (1986), Ultrastruct. Pathol., 10, 113-117). This protein has been shown to cause ulceration in mice (Telford, J.L. *et al.*, (1994), J. Exp. Med., 179, 1653-1658) and is a vaccine candidate. The purified cytotoxin is a protein of 87-94 kD that can be purified in very small amounts from bacterial culture supernatant.

10

Material and methods

Bacterial strain.

- 15 The *Helicobacter pylori* CCUG 17874 (type strain, Culture Collection, University of Goteborg) was used.

Media and supplements.

- 20 Brucella Broth (tryptone 10 g l⁻¹, peptamin 10 g l⁻¹, dextrose 1 g l⁻¹, yeast extract 2 g l⁻¹, sodium chloride 5 g l⁻¹ and sodium bisulfite 0.1 g l⁻¹) (BB)(Difco) supplemented with 2 g l⁻¹ of (2,6 di-O-methyl)- β -cyclodextrin (CD) (Teijin Lim. Tokyo, Japan) and 20 mg/L of streptomycin was used as liquid medium for comparison purpose. Hysoy or Soytone were used at a concentration of 10 g/L instead of tryptone and peptamin present in BB.

25

Preservation.

- Frozen aliquots for inocula were prepared from flask cultures of 2×10^8 CFU ml⁻¹ diluted 1:2 with a solution composed of glycerol 40%, fetal calf serum (FCS) (HyClone, Logan, Utah) 20% and 0.4% CD. The suspension obtained was distributed in 3 ml vials and stored at -80 °C and used as starting frozen vials for comparison with new frozen vials prepared substituting FCS with Soytone 20%.

Growth in liquid medium.

Initial cultures were performed in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium. Cultures were inoculated with 3 ml of frozen stocks and incubated at 36 °C for 36 hours with shaking (100 rpm, 2.5 cm throw) in a microaerobic environment. Flasks were placed inside an anaerobic jar where BBL Campy Pak envelopes (Becton Dickinson) were used to generate the proper conditions. These cultures were then used to inoculate 1000 ml flasks containing 250 ml of medium and incubated in the same conditions mentioned above and used to inoculate the bioreactors.

Culture vessels and growth conditions.

Batch fermentations were carried out in 7 litres bioreactors (MBR Bioreactors AG, 8620 Wetzikon, CH) containing 5 l of medium. All cultures were grown at 36°C. The pH values were not controlled. The dissolved oxygen tension (DOT) was maintained automatically at the pre-set level (3%) by a two step procedure. First, air flow rate was increased from 0.1 up to 0.5 l l⁻¹ min⁻¹ to satisfy the increasing O₂ demand of the culture. If further increases were necessary, they were obtained by supplying pure O₂ up to a maximum of 0.4 l l⁻¹ min⁻¹. During the first 12 hours of growth, a constant flow of N₂ and CO₂ was maintained equal to 0.2 l l⁻¹ min⁻¹ and 0.02 l l⁻¹ min⁻¹ respectively. The agitation speed was maintained at 130 rpm. The agitator shaft was equipped with two Rhuston turbines having a diameter of 7 cm and the diameter of the bioreactor was 17 cm.

Glucose feed.

A 50% glucose solution was added at time 0 to give a final concentration of 5 g/L. Another addition of 5 g/L was made when the OD was in a range 2-3.

Biomass determination.

Growth was monitored by optical density at 590 nm against a water blank (Perkin Elmer 35 spectrophotometer), light path of 1 cm. Purity checks of the samples were made by Gram staining.

5 Analysis of the VacA protein.

At determined time points during the fermentation, culture samples were centrifuged (Biofuge A, Heareus) at 8,300 x g per 10 min. The supernatants were precipitated with trichloroacetic acid and subjected to 9% SDS-Page using a BioRad Mini Protean II apparatus. Proteins were transferred to nitrocellulose filters (Schleicher & Schuell) and then incubated overnight with polyclonal antisera raised against the VacA protein (Telford, J.L. *et al.*, (1994), J. Exp. Med., 179, 1653-1658). After incubation for 2 hours with a horseradish-peroxidase conjugated secondary antibody (Sigma), the immunoreactive bands were visualized by 4-chloro-naphtol staining.

15

Results

Brucella Broth is a complex medium composed of tryptone 10 g l⁻¹, peptamin 10 g l⁻¹, dextrose 1 g l⁻¹, yeast extract 2 g l⁻¹, sodium chloride 5 g l⁻¹ and sodium bisulfite 0.1 g l⁻¹. This medium has been described in many articles as capable of supporting the growth of *H. pylori* only when supplemented with blood derivatives (Cover, T.L. and Blaser, M.J. (1992), J. Biol. Chem., 267, 10570-10575; Shahamat, M. *et al.*, (1991), J. Clin. Microbiol., 29, 2835-2837; Buck, G.E. and Smith, J.S. (1987), J. Clin. Microbiol., 25, 597- 599; and Morgan, D.R. *et al.*, (1987), J. Clin. Microbiol., 25, 2123-2125). A substantial simplification of *H. pylori* growth media was obtained recently when it was discovered that cyclodextrins could be used in the place of blood derivatives (Olivieri, R. *et al.*, (1993), J. Clin. Microbiol., 31, 160-162). The growth of *H. pylori* using this simplified medium and glucose feed is reported in Figure 1. Glucose feeds were used to ensure that the carbon and energy source was always present in the medium.

30

When *H. pylori* was cultured in these conditions, the production of VacA in the medium was measured as described above. The results are shown in Figure 5 and Figure 6.

The use of Soytone and Hysoy in the growth media gave the results reported in Figures 2 and 3 respectively. VacA production is reported in Figure 5 when Soytone was used and in Figure 6 when Hysoy was used.

5

The results show improvements to the growth media and fermentation conditions of *Helicobacter pylori* growth and in the production of the vacuolating cytotoxin (VacA).

Example II

10

Haemophilus influenzae are small, non motile, gram negative bacteria that are the major cause of bacterial meningitidis in children. These microorganisms are primarily invasive rather than toxigenic. They are inhabitants of the respiratory tract (commensal as well pathogenic) and they have antiphagocytic polysaccharides capsules.

15

Materials and methods

Bacterial strain

20 *Haemophilus influenzae* B ATCC 10211

Media and Supplements

The preparation of the media, involves use of different solutions as follows:

25

"Franz A medium" Preparation

30

<u>Component</u>	<u>Amount per litre</u>
Purified water	800 mL
Glutamic acid	1,6 g +/- 0.01 g
Na ₂ HPO ₄ 12H ₂ O	5.03 g +/- 0.05 g
KCl	0.892 g +/- 0.008 g

14

NaCl	6.005 g +/- 0.06 g
NH ₄ Cl	1.25 g +/- 0.01 g
Purified water	QS to 1.0 litre
3N NaOH	as required for pH = 8.2.

5

The above components are dissolved with mixing. 3N NaOH is used to pH the solution to pH = 8.2

Ultrafiltered Soytone preparation

10

<u>Component</u>	<u>Amount per litre</u>
Soytone	33.3 g +/- 0.03 g
Purified water	QS to 1.0 litre

15

This solution was ultrafiltered through a 30 kD TFF apparatus.

Ultrafiltered Proteose Peptone

20

<u>Component</u>	<u>Amount per litre</u>
Proteose peptone	33.3 g +/- 0.03 g
Purified water	QS to 1.0 litre

This solution was ultrafiltered through a 30 kD TFF apparatus

25

Ultrafiltered YE preparation

<u>Component</u>	<u>Amount per litre</u>
yeast extract	100 g +/- 0.02 g
Purified water	QS to 1.0 litre

30

The yeast extract used was HY YEST available from Quest. The solution was

15

ultrafiltered through a 10 kD TFF apparatus.

50% Glucose Solution

5	<u>Component</u>	<u>Amount per litre</u>
	glucose (anhydrous)	500 g +/- 5 g
	Purified water	QS to 1.0 litre

10 NAD 0.1 % solution

	<u>Component</u>	<u>Amount per litre</u>
	NAD	1.0 g +/- 0.005g
	TRIS	1.21g +/- 0.01g
15	Purified water	QS to 1.0 litre
	HCl 37%	as required to pH 7.4 \pm 0.2

Hemin 0.4% solution

20	<u>Component</u>	<u>Amount per litre</u>
	Hemin	4 g +/- 0.02g
	0.2N NaOH	QS to 1.0 litre

The hemin used is preferably chemically synthesised. Chemically synthesised hemin
25 is commercially available from Fluka.

550 ml of Franz A was mixed with 450 ml of ultrafiltrated soytone or Proteose
Peptone to obtain one litre of Hib basal medium which was sterilized by autoclaving at
121 °C for 30 minutes.

30

After cooling, 10 ml/L glucose solution, 20 ml/L of YE, 2 ml/L NAD solution,
sterilized by filtration, were added (with the additions mentioned before) and the

medium called "Hib Complete medium".

Growth in liquid media

- 5 Pre-warmed, unbaffled shake flasks (500/150) were inoculated with 1.0 mL of a working stock vial each. The shake flasks were placed in an (1 inch throw) incubator-shaker at 35 +/- 1° C at 150 RPM for 6 hours.

After 6 hours, the appropriate amount of the shake flask was transferred into one 2 L
10 litre unbaffled shake flask containing 0.5 L of pre-warmed "Hib complete medium". The shake flask was placed in an (1 inch throw) incubator-shaker at 35 +/- 1° C at 200 rpm for 9 hours then the content was transferred into a sterile inoculation and the inoculate then transferred to the fermenter.

15 Culture vessels and growth conditions

Batch fermentations were carried out in 30 litres bioreactors (MBR Bioreactors AG, 8620 Wetzikon, CH) containing 20 l of medium.

The cultures were growing at 35 °C and 2 psi back pressure. The pH was controlled to
20 7.3 with 3 N NaOH. The initial agitation rate was set at minimum of 150 rpm, and bottom aeration at 10 L/minute. DOT was maintained at 35% by rpm control in a range 150-400, then supplementing with oxygen if necessary. Antifoam was added manually to control foaming. Residual concentration of glucose was detected and when it was around 2 g/L, 0.2 litres of glucose solution were added.

25

Biomass determination.

Growth was monitored by optical density at 590 nm against a water blank (Perkin Elmer 35 spectrophotometer), light path of 1 cm. Purity checks of the samples were
30 made by Gram staining.

Analysis of Hib PS.

This analysis was performed by Rocket immuno electrophoresis as described by Weeke, B., Scand. J. Immunol. 2, 37-46, (1973), herein incorporated by reference.

5 Results

The growth curves obtained with Soytone and with Proteose peptone are compared in Figure 7. The yield of Hib PS was 600 mg/L and 150 mg/L in the media containing Soytone and Proteose peptone respectively. Although the growth curves are quite similar using the two media, there is a fourfold increase in the yield of Hib PS using
10 Soytone. The use of the culture media containing the vegetable derived proteinaceous material leads to an increased yield of polysaccharides, such as Hib PS, compared to the use of a culture medium containing animal derived proteinaceous material.

Example III

15

C. diphtheriae are gram positive, rod like microorganisms, which arrange themselves in palisades. *C. diphtheriae* lysogenization by a bacteriophage causes the synthesis of a potent toxin whose expression is regulated by iron concentration.

20 Materials and methods

Bacterial strain

C. diphtheriae CN 2000

25

Media and supplements

The preparation of the media, involves use of different solutions as follows:

30 A. Yeast Extract (YE) and Casamino Acids (CAA) Ultrafiltered

<u>Component</u>	<u>Amount g/L</u>
------------------	-------------------

18

Purified water	800 ml
Yeast Extract	20 g/L
Casamino Acids	10 g/L
Purified water	QS to 1 L

5

This solution was ultrafiltered through a 10 kD TFF apparatus and the permeate added to the deferration vessel.

10 A.A Yeast Extract (YE) and Soytone Ultrafiltered

	<u>Component</u>	<u>Amount g/L</u>
	Purified water	800 ml
	Yeast Extract	20 g/L
15	Soytone	10 g/L
	Purified water	QS to 1 L

This solution was ultrafiltered through a 10 kD TFF apparatus and the permeate added to the deferration vessel.

20

A.B Yeast Extract Ultrafiltered

	<u>Component</u>	<u>Amount g/L</u>
	Purified water	800 ml
25	Yeast Extract	30 g/l
	Purified water	QS to 1 L

This solution was ultrafiltered through a 10 kD TFF apparatus and the permeate added to the deferration vessel.

30

A.1 Deferration

The following components were introduced into an agitated vessel

	<u>Component</u>	<u>Amount</u>
	UF (YE + CAA) solution	1 L
5	KH ₂ PO ₄	5 g/L
	CaCl ₂ ·2H ₂ O, 50% (w/v)	2 mL/L
	L-tryptophan, 1% (w/v)	5 mL/L (0.05 g/L)
	3N NaOH	(enough to correct pH to 7.4)

10 UF indicates that the solution is ultrafiltered.

With agitation, heat the solution to 100 °C. Hold at 100 °C for 1 minute, then cool medium to 37 °C. Filtration can commence once below 37 °C. After filtration, the medium is described as "CY base medium with CAA".

15

A.1.A Deferration

The following components were introduced into an agitated vessel

	<u>Component</u>	<u>Amount</u>
20	UF (YE + Soytone) solution	1 L
	KH ₂ PO ₄	5 g/L
	CaCl ₂ ·2H ₂ O, 50% (w/v)	2 mL/L
	L-tryptophan, 1% (w/v)	5 mL/L (0.05 g/L)
25	3N NaOH	(enough to correct pH to 7.4)

With agitation, heat the solution to 100 °C. Hold at 100 °C for 1 minute, then cool medium to 37 °C. Filtration can commence once below 37 °C.

After filtration, the medium is described as "CY base medium with Soytone".

30

A.1.B Deferration

The following components were introduced into an agitated vessel

	<u>Component</u>	<u>Amount</u>
	UF YE solution	1 L
5	KH ₂ PO ₄	5 g/L
	CaCl ₂ -2H ₂ O, 50% (w/v)	2 mL/L
	L-tryptophan, 1% (w/v)	5 mL/L (0.05 g/L)
	3N NaOH	(enough to correct pH to 7.4)

10 With agitation, heat the solution to 100 °C. Hold at 100 °C for 1 minute, then cool medium to 37 °C. Filtration can commence once below 37 °C. After filtration, the medium is described as "CY base medium with YE only".

B. "Supplements" solution

15

B1) Solution A

	<u>Component</u>	<u>Amount per litre</u>
20	MgSO ₄ - 7 H ₂ O	225 g
	Beta-alanine	1.15 g
	Nicotinic acid	1.15 g
	Pimelic acid	0.075 g
	CuSO ₄	0.50 g
25	ZnSO ₄ - 7 H ₂ O	0.40 g
	MnCl ₂ - 4 H ₂ O	0.15 g
	HCl, 37%	30 mL
	Purified water	QS to 1.0 litres

30 B2) Solution B

<u>Component</u>	<u>Amount per litre</u>
------------------	-------------------------

21

Purified water	800 mL
L-cystine	200 g
HCl, 37%	200 mL

- 5 The solutions are mixed individually for 10 minutes. After dissolution, 20 mL of Solution A and 10 mL of Solution B are mixed and filtered through a 0.2 micron filter. The solution is stored at 4 °C covered from light.

Flasks Sterilization

10

Once deferrated, filtered media (A.1 or A.1.A or A.1.B) are loaded into the flasks. Sterilize the flasks for 25 minutes at 121 °C.

Post-Sterilization Adjustments

15

1. Add "50% Maltose" 30 ml/L
2. Add "Supplements" 3.0 ml/L

Media with 1 + 2 above: "Complete CY medium with CAA or with Soytone or with YE

20 *only"*

Growth in liquid media

500 ml unbaffled shake flasks each with 100 ml of medium, were inoculated with 1.0
25 mL of a working stock vial.

The shake flasks were placed in an (1 inch throw) incubator-shaker at 35 +/- 1°C at 100 rpm for 5 hours. After 5 hours, the agitation is increased to 250 rpm for 43 hours.

30

The process has two distinct phases: 1) Exponential growth), and 2) Production phase. The transition from the two phases is gradual and is marked by reductions in cell

growth rate and oxygen demand.

Biomass determination.

- 5 Growth was monitored by optical density at 590 nm against a water blank (Perkin Elmer 35 spectrophotometer), light path of 1 cm. Purity checks of the samples were made by Gram staining.

Results

10

After 48 hours of incubation, the OD and the Lf/ml in the flasks were:

	medium with CAA.	OD = 7.5	Lf/ml = 50
	medium with Soytone.	OD = 7.87	Lf/ml = 60
15	medium with YE only	OD = 8.07	Lf/ml = 60

Example IV

- 20 *Neisseria meningitidis* are non motile gram negative cocci, most often growing in pairs but occasionally in tetrads or clusters. They have antiphagocytic polysaccharides capsules which is the basis for serogroups.

Materials and methods

25

Bacteria strain

Neisseria meningitidis. C11

30 Media and supplements

Preparation of the media involves use of different solutions as follows:

"Franz medium" Preparation**Inoculum Shake Flask Medium Preparation**

5	<u>Component</u>	<u>Amount per litre</u>
	Purified water	800 mL
	Glutamic acid	1,6 g
	Na ₂ HPO ₄ ·2H ₂ O	15.5 g
	KCl	0.09 g
10	NH ₄ Cl	1.25 g
	Purified water	QS to 1.0 litre
	3N NaOH	as required for pH = 7.6

Dissolve the above components with mixing. Sterilize by autoclaving at 121 °C, 30 minutes. After cooling, the "50% Glucose" and "Men C Supplements" are added as below.

"50% Glucose"	10 mL/L +/- 0.3 mL
"Men C Fermentation Supplements"	20 mL/L +/- 0.3 mL

20

With the addition of the glucose and supplements solutions, the medium can be called "Franz Complete medium".

Preparation of the 20 L fermenter

25

A. The following components must be introduced to a mixing vessel and dissolved to create 20 L of Watson base medium with CAA:

	<u>Component</u>	<u>Amount per litre</u>
30	Purified water	800 mL
	Glutamic acid	1 g +/- 0.01 g
	Na ₂ HPO ₄ ·2H ₂ O	3.25 g +/- 0.03 g

24

KCl	0.09 g +/- 0.001 g
Casamino acid	10 g +/-0.1 g
Purified water	QS to 1.0 litre
3N NaOH	as required for pH = 7.6

5

A.A The following components must be introduced to a mixing vessel and dissolved to create 20 L of Watson base medium with Amisoy:

	<u>Component</u>	<u>Amount per litre</u>
10	Purified water	800 mL
	Glutamic acid	1 g +/- 0.01 g
	Na ₂ HPO ₄ 2H ₂ O	3.25 g +/- 0.03 g
	KCl	0.09 g +/- 0.001 g
	Amisoy	10 g +/-0.1 g
15	Purified water	QS to 1.0 litre
	3N NaOH	as required for pH = 7.6

B. Fermenter Sterilization

20

Sterilize the fermenter for 25 minutes at 121 °C. After sterilization, set the following conditions: temperature, 35 °C; aeration at 10 L/minute; agitation at 150 rpm, and backpressure at 0.2 bar. After cooling the fermenter to 35 °C, and take a sample to measure pH, adjust the fermenter pH probe calibration and then adjust the medium pH to 7.6 .

C. Post-Sterilization Adjustments

1. Add "50% Glucose" 10 ml/L
- 30 2. Add "Men C Supplement" 20 ml/L

Medium with 1 + 2 above: "Watson Complete medium "

A. "50% Glucose" Solution

	<u>Component</u>	<u>Amount per litre</u>
5	glucose (anhydrous)	500 g +/- 5 g
	Purified water	QS to 1.0 L

This solution is sterilized by autoclaving at 121 °C for 30 minutes.

10 B. "Men C Supplements" solution

B.1 Ultrafiltered YE

	<u>Component</u>	<u>Amount</u>
15	Purified water	QS to 1 L
	Yeast Extract	125 g/L

This solution is ultrafiltered through a 10 kD TFF apparatus.

The retentate is discarded after the requisite permeate has been collected, and the
20 permeate is added to the vessel containing the supplement.

B.2

	<u>Component</u>	<u>Amount per litre of UF YE</u>
	MgSO ₄ - 7 H ₂ O	30 g +/- 0.5 g
25	L-cystein-HCl	1.5 g +/- 0.2 g

Add the chemicals to 1 L UF YE, mix for 10 minutes, filter sterilized through a 0.2 micron filter and transfer to the fermenter.

C. 3 N NaOH

30

3 N NaOH = 12 % (w/v) NaOH = 120 g/L NaOH

D. Antifoam

The antifoam used is Dow Corning 1510. It is sterilized by autoclaving at 121 °C for 30 minutes

5 Growth in liquid media

A 500 millilitre flask is inoculated with 1.0 mL of a working stock vial. The shake flask contains 150 mL of complete "Franz medium".

- 10 The inoculated shake flasks is placed in an (1 inch throw) incubator-shaker at 35 +/- 1 °C at 150 rpm. After 10 hours, the flask is aseptically sampled. The optical density should be between 1.3 - 3.3 (at 590 nm) if so, the four 2 L shake flasks are each inoculated with the appropriate volume from the 150 mL shake flask.

- 15 Each 2 L shake flask contains 0.2 L of pre-warmed "Franz Complete medium".

The shake flasks are placed in an (1 inch throw) incubator-shaker at 35 +/- 1 °C at 200 rpm. After 10 hours, the contents of each shake flask are transferred into the sterile inoculation can and inoculate the fermenter.

20

Culture vessel and growth conditions

Batch fermentations were carried out in 30 litres bioreactors (MBR Bioreactors AG, 8620 Wetzikon, CH) containing 20 L of medium.

25

The cultures were growing at 35 °C and 14×10^3 N/m² (2 psi) back pressure. The pH was controlled to 7.3 with 3 N NaOH. The initial agitation rate was set at minimum of 150 rpm, and bottom aeration at 10 L/minute. DOT was maintained at 35% by rpm control in a range 150-400, then supplementing with oxygen if necessary. Antifoam

- 30 was added manually to control foaming. Residual concentration of glucose was detected and when it was around 2 g/L, 0.2 litres of glucose solution were added.

Biomass determination.

Growth was monitored by optical density at 590 nm against a water blank (Perkin Elmer 35 spectrophotometer), light path of 1 cm. Purity checks of the samples were made by Gram staining.

Analysis of MenC PS

Quantitative estimation of the polysaccharides was performed analyzing the sialic acid content according to the method reported in Biochimica and Biophysica Acta (1957), 21, 610, by Lars Svennerholm.

Results

The growth curves obtained with Amisoy and with Casamino acids are compared in Figure 8. The yield in MenC PS was 307 mg/L and 345 mg/L in the media containing Amisoy and Casamino acids respectively. The growth curves and PS production are quite similar using the two media.

It will be understood that the invention is described above by way of example only and modifications may be made within the scope of the invention as defined in the appended claims.

Example V

25

Clostridium tetani is a slender bacillus measuring 2µm in length and 0.3-0.5µm in width. It often exists in the form of a rather long filament-like cell. When spores are formed, the bacillus assumes the characteristic drumstick appearance. It is a mobile organism, gram positive, but its gram stain can become variable or even negative in aging cultures. *Clostridium tetani* is a strict anaerobe and produces two exotoxins. One of these, the tetano spasmin, is a neurotoxin responsible for the whole clinical picture of the disease.

Materials and methods

Bacterial strain

5

Clostridium tetani Harvary Y – VI-3.

Media

10 The seed cultures were prepared using the medium reported below expressed in g/L:

<u>Component</u>	<u>Amount g/L</u>
N-Z Soy	15.0
Glucose	5.5
Yeast extract	5.0
NaCl	2.5
L-Cysteine	0.5
Sodium thioglycollate	0.5
Agar	0.75
PH = 7.1	

The production media were prepared as modification of the Mueller-Miller medium described in the WHO/VSQ/GEN/94 (1990). In this medium, beef heart infusion and
 15 casein solution are used, in the modified media reported below, expressed in g/L,
 Hysoy and Soytone were used instead of beef heart infusion and casein solution:

<u>Component</u>	<u>Amount g/L</u>
Glucose – H ₂ O	12.1
NaCl	2.5
Na ₂ HPO ₄ -12H ₂ O	2.5
KH ₂ PO ₄	0.15
MgSO ₄ -7H ₂ O	0.15
Amino acids solution	17.5 ml

29

Vitamine solution	4.2 ml
NaOH 5M	4.0 ml
FeSO ₄ -7 H ₂ O (1% sol.)	4.0 ml
Soy derivatives	20.0
PH = 7.3	

Sterilised by autoclaving at 120°C for 20 min

Amino acids solution:

5

<u>Component</u>	
L-Tyrosine	28.51 g/L
Uracil-	0.142 g/L
L- Cystein-	14.25 g/L
HCl 37%	131.6 ml/L

Vitamin solution:

<u>Component</u>	
Ca pantithenate	238.1 mg/L
Thiamine	59.7 mg/L
Pyridoxin	59.7 mg/L
Riboflavin	59.7 mg/L
Biotin	0.73 mg/L
Ethanol	256.4 ml/L

Growth in liquid media

- 10 Two 25 ml tubes, containing 15 ml of seed medium, were inoculated with 0.5 ml each of working seed vial and incubated at 35°C for 29 hrs in anaerobic jar where a Gas generating kit (OXOID) was used. A second series of tubes were inoculated by 1.5 ml of the first tubes and incubated in the same conditions reported above for 24 hrs.

7 ml of these cultures were used to inoculate 100 ml tubes containing 75 ml of the same medium. These tubes were incubated in the same conditions reported above for 24 hrs.

- 5 The entire content of these tubes were used to inoculate 5000 ml beakers containing 2500 ml of the production medium.

Biomass determination

- 10 Growth was monitored by optical density at 590 nm against a water blank (Pharmacia spectrophotometer), light path of 1 cm. Purity checks of the samples were made by Gram staining.

Results

15

After 186 hrs of incubation, the OD and the Lf/ml in the breakers were:

Medium with Hysoy	OD = 1.08	Lf/ml = 60
Medium with Soytone	OD = 0.74	Lf/ml = 60
Medium with beef heart infusion and casein solution	OD = 1.236	Lf/ml = 60,

Example VI

20

Bordetella pertussis is a gram negative coccobacillus about 0.5µm in diameter and 0.5 to 2µm in length. Its nutritional requirements are simple, and it does not utilize sugars. It is extremely sensitive to fatty acids and survives poorly without protective factors.

25 Materials and methods

Bacterial strain

Bordetella pertussis 9K/129G (Pizza, M., et al. (1989) Science, 246, 497-500).

Media

The seed and production cultures were prepared using CL medium (Imaizumi, A., *et al.* (1983) *Infection and Immunity*, 41 (3), 1138-1143) reported below expressed in g/L:

<u>Component</u>	<u>Amount g/L</u>
Sodium L-glutamate	10.7
L-proline	0.24
NaCl	2.5
KH ₂ PO ₄	0.5
KCl	0.2
MgCl ₂ - 6H ₂ O	0.1
CaCl ₂	0.02
Tris	6.1
L-Cysteine*	0.04
FeSO ₄ - 7 H ₂ O*	0.01
Niacin*	0.004
Glutathione reduced*	0.15
Ascorbic acid*	0.4
Casaminoacid	10.0
Dimethyl-B-cyclodextrin	1.0

PH adjusted to 7.6 with HCl.

10 * sterilized by filtration and then added aseptically to the autoclaved medium

The modified medium contained 10 g/l of N-Z soy instead of Casaminoacid.

Growth in liquid media

15 500 ml unbaffled shake flasks each with 100 ml of CL medium or the modified one, were inoculated with 3.0 mL of a working stock vial.

The shake flasks were placed in an (1" throw) incubator-shaker at 35 +/- 1°C at 100 RPM for 12 hours. After 12 hours, the agitation was increased to 250 RPM for another 16 hours.

5

Biomass determination

Growth was monitored by optical density at 590 nm against a water blank (Pharmacia spectrophotometer), light path of 1 cm. Purity checks of the samples were made by
10 Gram staining.

Analysis of PT

This analysis was performed by ELISA (Nencioni, L., *et al.* (1990) Infect. Immun.,
15 58, 1306-1315).

Results

After 28 hrs of incubation, the OD and PT (mg/L) in the flasks were:

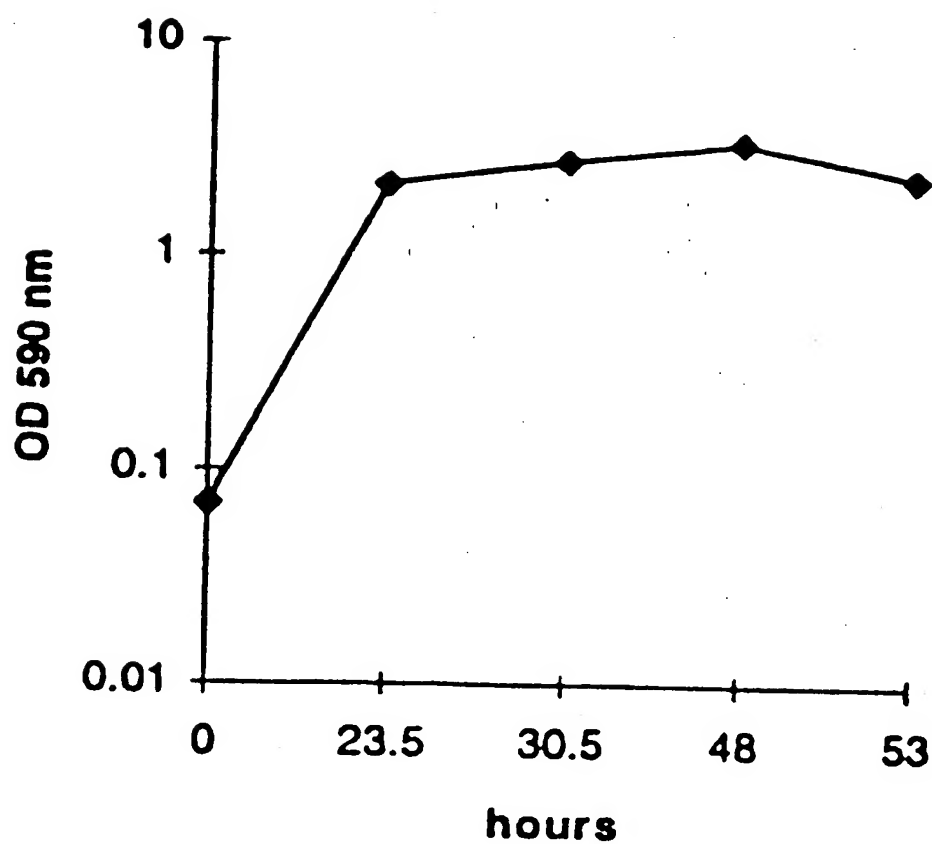
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medium with CAA.	OD = 2.31	PT = 2.4 mg/L
Medium with NZ-soy.	OD = 1.99	PT = 2.25 mg/L

Claims

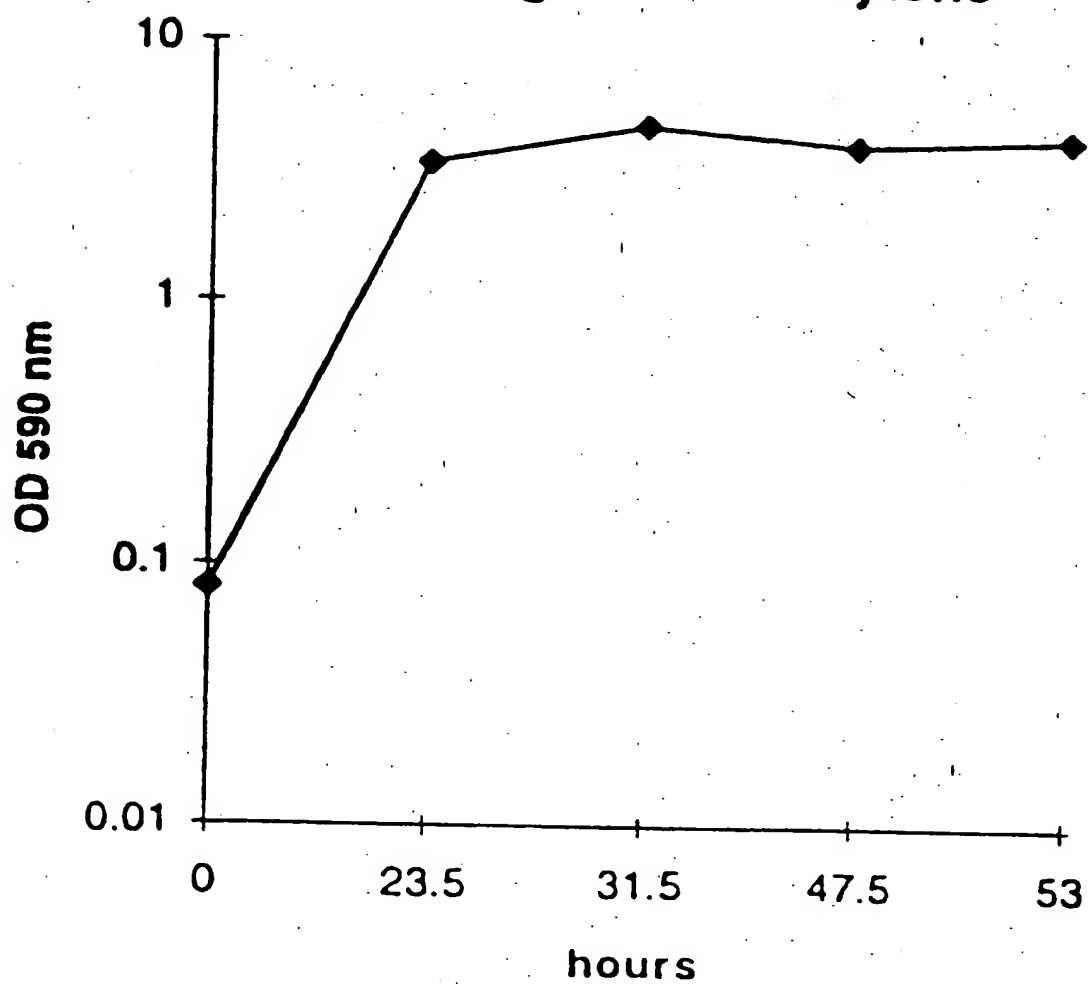
1. A medium for cultivating pathogenic bacteria to produce an immunogenic factor wherein the medium comprises at least 20% by dry weight of a non-animal derived proteinaceous material, and does not comprise animal derived proteinaceous material.
5
2. The medium of claim 1 wherein the non-animal derived proteinaceous material is a yeast extract and/or a soy bean derived protein composition.
- 10 3. The medium of claim 1 or claim 2 wherein the pathogenic bacteria are *Helicobacter pylori*, *Haemophilus influenzae*, *Corynebacterium diphtheriae*, *Neisseria meningitidis*, *Bordetella pertussis* or *Clostridium tetani*.
4. A culture comprising the medium of any one of the previous claims and
15 pathogenic bacteria.
5. A process for preparing an immunogenic factor of a pathogenic bacteria comprising the steps of cultivating the bacteria in the medium of any one of claims 1 to 3 and optionally purifying the immunogenic factor from the medium.
20
6. A process for the production of a vaccine comprising preparing an immunogenic factor of a pathogenic bacteria by the process of claim 5, and bringing said factor, optionally toxoided, into association with a pharmaceutically acceptable carrier.
- 25 7. A process for making a medium for cultivating pathogenic bacteria to produce an immunogenic factor comprising adding sufficient non-animal derived proteinaceous material to a standard medium for cultivating bacteria, which does not comprise animal derived proteinaceous material, so that the medium for cultivating pathogenic bacteria comprises at least 20% by dry weight of the non-animal derived proteinaceous material,
30 and does not comprise animal derived proteinaceous material.

1 / 8

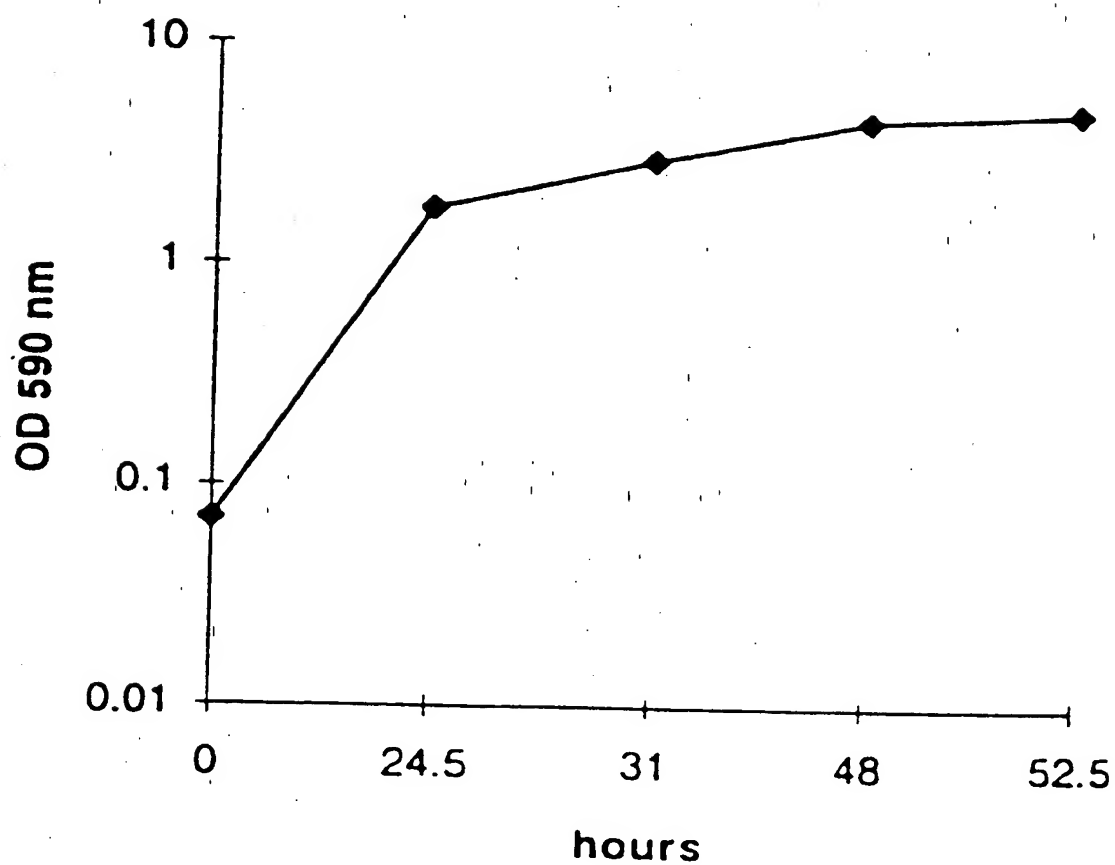
FIG. 1**H.pylori growth in BB medium**

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FIG. 2
H.pylori growth in Soytone



3/8

FIG. 3**H.pylori growth in HY soy**

4/8

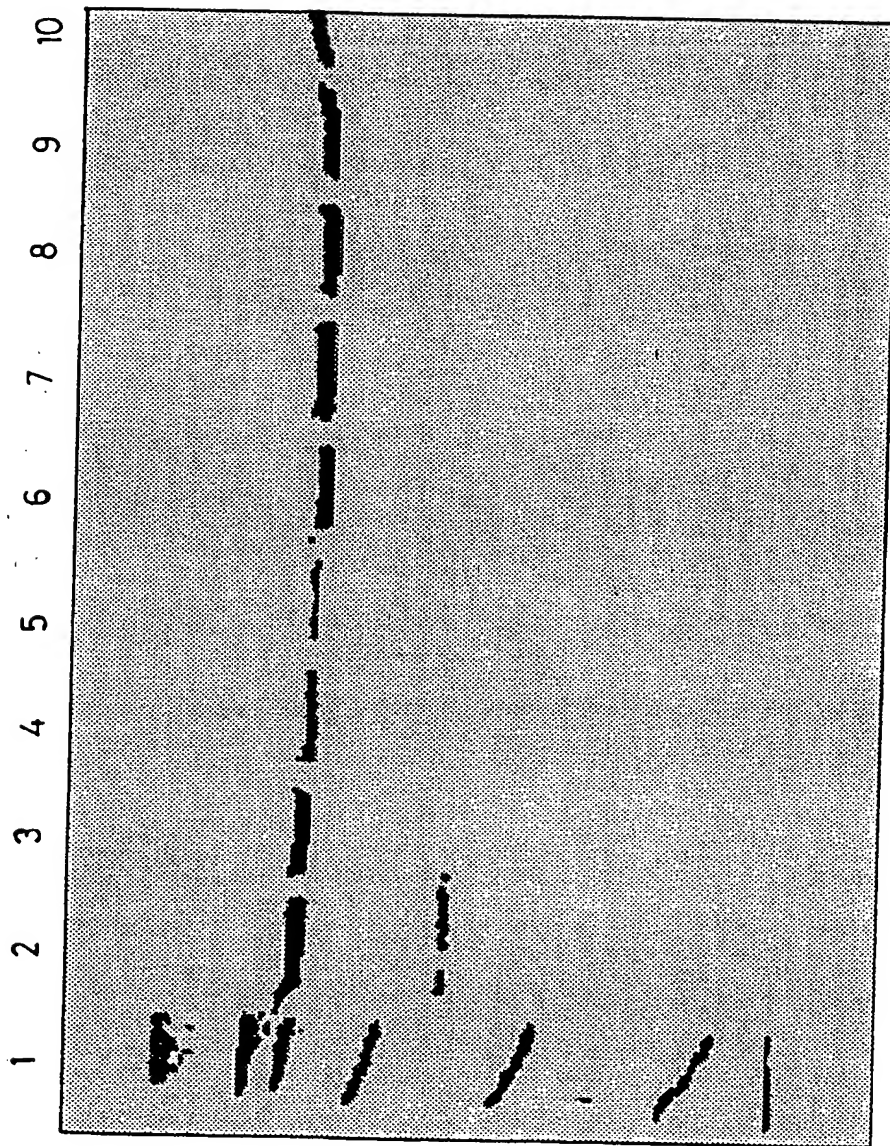


FIG. 4

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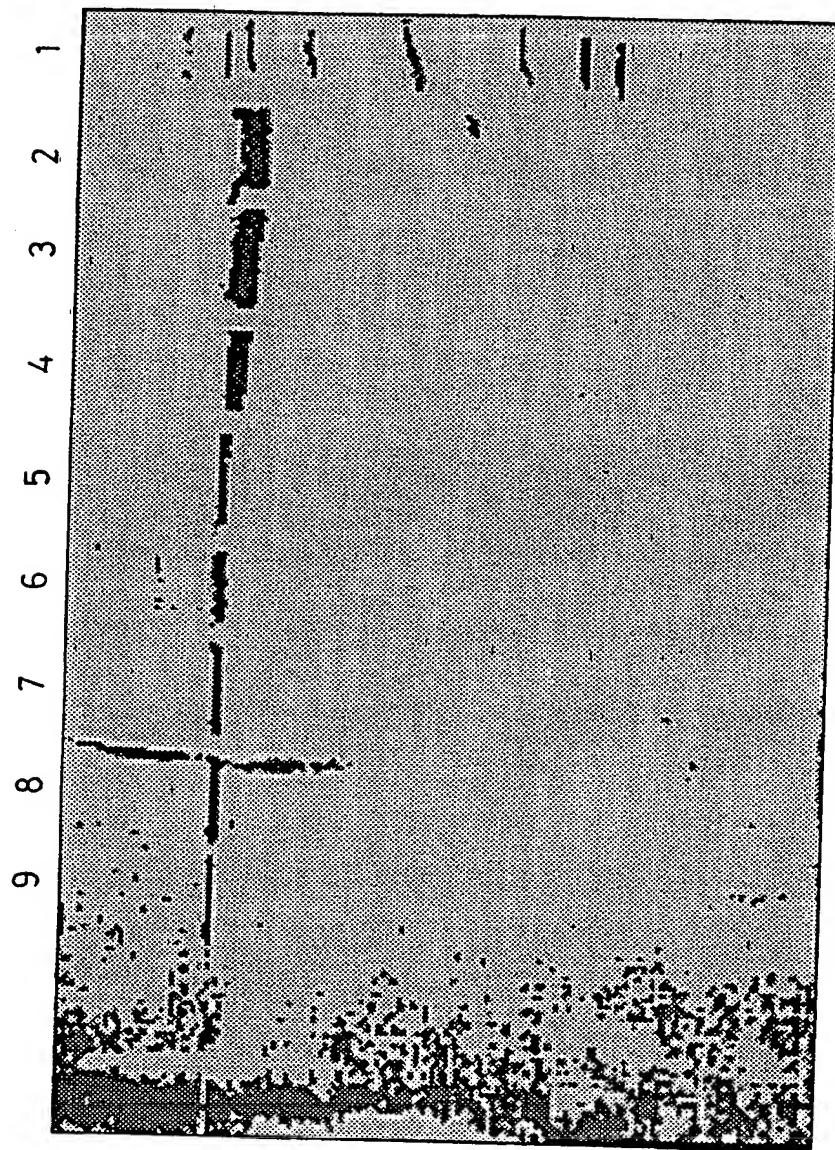


FIG. 5

6 / 8

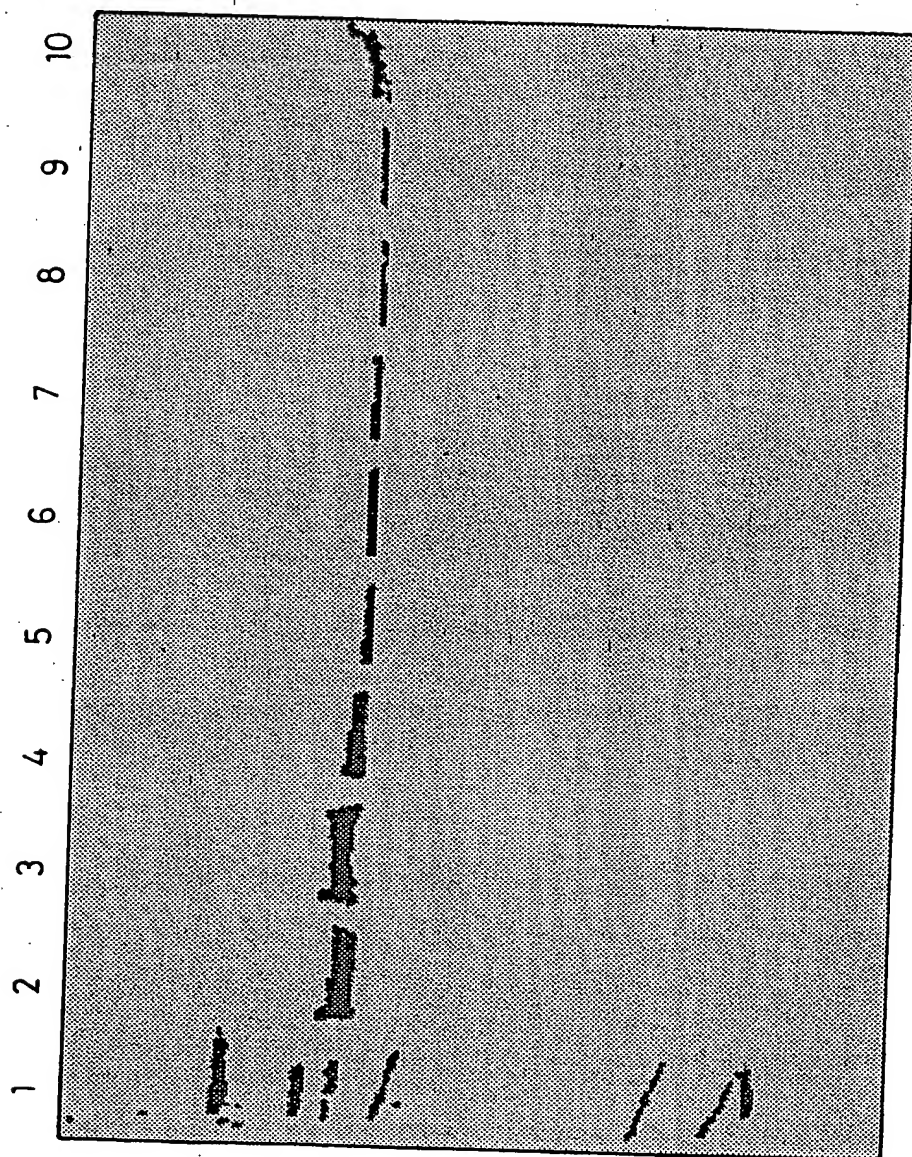
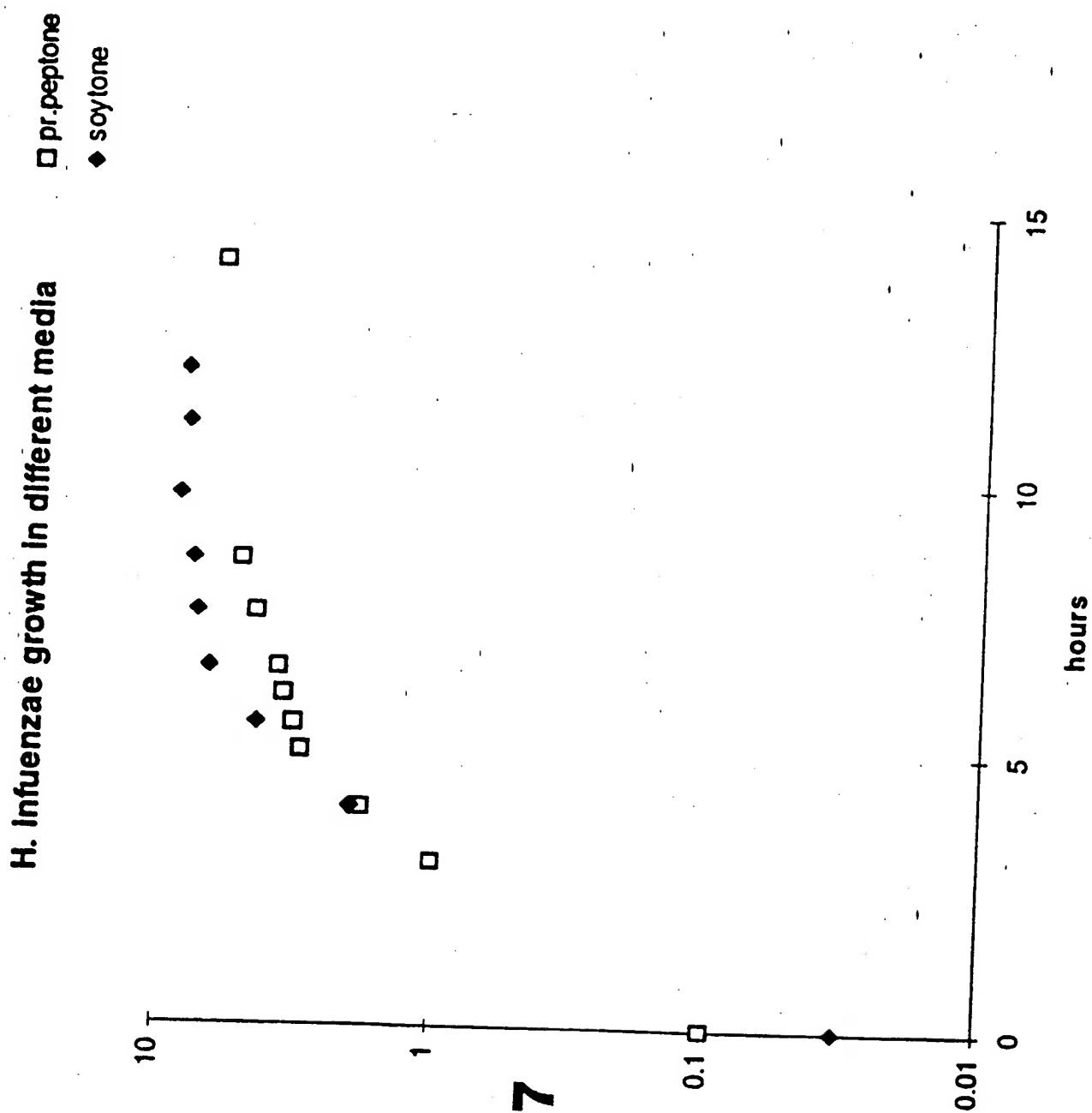
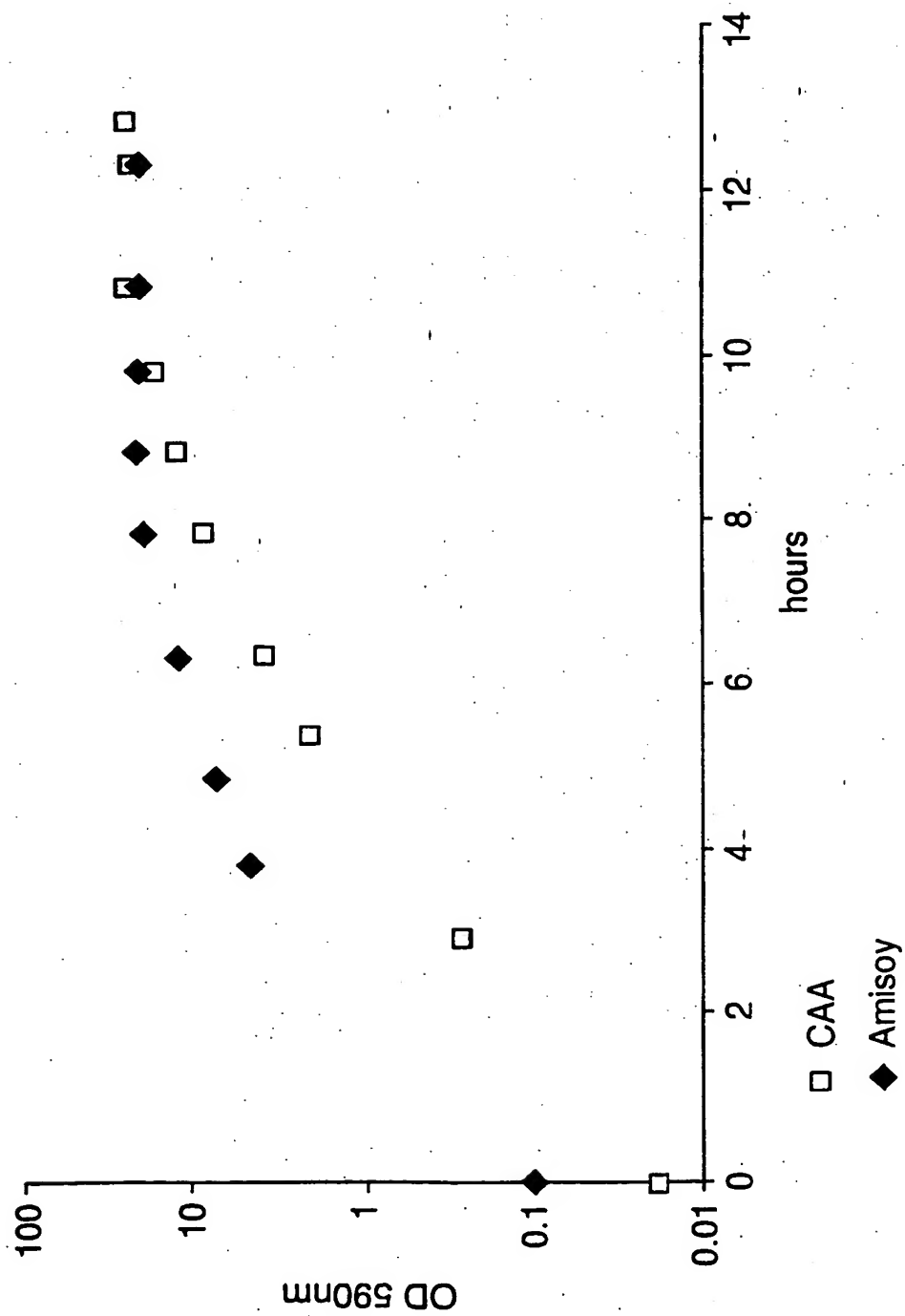


FIG. 6

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**FIG. 7**

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FIG. 8*N. meningitidis* growth in different media

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 98/00938

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 09115 A (MERCK & CO INC) 28 April 1994 see the whole document	1,2,4-7
X	EP 0 540 897 A (ISTITUTO RICERCHE IMMUNOBIOLOG) 12 May 1993 see abstract see page 2, column 1, line 1 - line 46 see page 2, column 2, line 1 - line 29 see examples 1,2	1,3-7
X	EP 0 575 951 A (MUELLER KARL & CO KG) 29 December 1993 see abstract see page 2, line 49 - line 51 see page 3, line 21 - line 28 see tables see example 2	1,2,4-7

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

9 November 1998

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INTERNATIONAL SEARCH REPORT

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PCT/IB 98/00938

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 494 808 A (FU JEFFREY) 27 February 1996 see abstract see column 3, line 46 - column 4, line 9 see column 5, line 1 - line 56 see table 1 ---	1,5-7
A	WO 96 14393 A (BIOCINE SPA) 17 May 1996 see abstract see page 3, line 21 - line 31 see page 4, line 6 - page 5, line 5 ---	1-7
A	MARCHINI A. ET AL.: "Cyclodextrins for growth of Helicobacter pylori and production of vacuolating cytotoxin" ARCHIVES OF MICROBIOLOGY, vol. 164, no. 4, 1995, pages 290-293, XP000560403 see the whole document ---	1-7
A	MARCHINI A. ET AL.: "Optimized conditions for the fermentation of Helicobacter pylori and production of vacuolating cytotoxin" FEMS MICROBIOLOGY LETTERS, vol. 124, no. 1, 1994, pages 55-59, XP000560409 see the whole document -----	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 98/00938

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WO 9614393 A	17-05-1996	EP 0789754 A JP 10500864 T	20-08-1997 27-01-1998

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